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Quality Review Procedures Necessary for Rodent Pathology Databases and Toxicogenomic Studies: The National Toxicology Program Experience

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ABSTRACT

Accuracy of the pathology data is crucial since rodent studies often provide critical data used for setting human chemical exposure standards. Diagnoses represent a judgment on the expected biological behavior of a lesion and peer review can improve diagnostic accuracy and consistency. With the conduct of 500 2-year rodent studies, the National Toxicology Program (NTP) has refined its process for comprehensive review of the pathology data and diagnoses. We have found that careful judgment can improve and simplify the review, whereas simply applying a set review procedure may not assure study quality. The use of reviewing pathologists and pathology peer review groups is a very effective procedure to increase study quality with minimal time and cost. New genomic technology to assess differential gene expression is being used to predict morphological phenotypes such as necrosis, hyperplasia, and neoplasia. The challenge for pathologists is to provide uniform pathology phenotypes that can be correlated with the gene expression changes. The lessons learned in assuring data quality in standard rodent studies also applies to the emerging field of toxicogenomics.

Keywords. Cancer; bioassay; rodent studies; F344 rat; B6C3F1 mouse; toxicology; toxicogenomics.

INTRODUCTION

Rodent studies, in the absence of definite results concerning humans, often provide critical data used by regulatory agencies in determining the potential hazard of a chemical, and for setting exposure standards (5, 22, 27, 28). International agencies, including components of the World Health Organization, also use NTP data, as well as data from studies conducted in many countries. The accuracy of the pathology evaluation is crucial to the outcome of toxicity and carcinogenicity studies and to the conclusions drawn from these studies (22). Using consistent terminology and diagnostic criteria for rodent lesions improves accuracy and facilitates comparison between studies (10). Long-term rodent studies are large complex research endeavors with multiple critical steps between the live animal and the final pathology tables. There is ample opportunity for errors to occur (9, 17). In the past 20 years, and with the conduct of nearly 500 separate 2-year rodent studies, the NTP has evolved a process to ensure accuracy of the pathology data.

Standard rodent studies, and more recently studies in transgenic rodents (19, 21), have been used to assess toxicity of chemicals. There has been a remarkable improvement in these studies, assisted in part, by the pathology peer review process. However, the application of functional genomics to toxicology (toxicogenomics), is rapidly changing toxicology and will greatly impact pathology and the role of pathologists (1). The pathologist can play a critical role in the genomic revolution. As in the past, accurate and consistent morphological characterization will be crucial. This manuscript describes processes and procedures that have been used to ensure pathology quality in toxicology studies and how this will be applicable to toxicogenomics.

REVIEW OF PATHOLOGY DATA AND MATERIALS

The review of the pathology data begins with an examination of the study protocol, clinical signs, gross observations and the records associated with slide preparation and the wet tissues. While the slides and diagnoses can be repeatedly evaluated, many of these early data points, unless carefully captured, can not be reconstructed later. Inclusion of all gross lesions, proper tissue collection and having complete records are often crucial for study interpretation. Their significance is easily overlooked in the multitude of tasks needed to be accomplished in a limited time during the final necropsy.

The NTP has found that a third party examination of wet tissues from 10% of the animals selected at random is crucial to identify potential lesions that may have been missed in the necropsy (17). The wet tissues and carcasses from all animals need to be saved until this process is completed. Two examples serve to illustrate this point. In one study, a slight increase (not significant) in mammary gland fibroadenomas in female rats prompted a review of the carcasses of all rats since this was a potential target tissue. Six additional mammary gland fibroadenomas were found in the controls and only one in the high exposure group suggesting that an exposure effect was not present (25). It was found that the study pathologist had focused on visceral lesions and not conducted a thorough examination of the whole carcass at necropsy review. In another study, a trihalomethane structurally related to bromodichloromethane known to cause colon tumors in male rats (7), was not found to cause colon tumors in male rats. A routine wet tissue review did not reveal untrimmed

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lesions. A more focused review revealed that contrary to the protocol, the very terminal end of the colon had not been opened and evaluated. Correcting this deficiency revealed a low incidence of colon tumors (24). Verification that all tissues are properly trimmed is a critical part of the study that needs to be done before any materials are discarded.

Ten percent of the glass slides are routinely reviewed for the presence of required tissues, and the tissues are trimmed according to protocol. For example, in NTP studies a single cross section of the trachea with paired thyroid lobes is the routine section for thyroid and parathyroid. The importance of uniform trimming of tissues was shown by an unexpected study result. Penicillin administration was found to be associated with an increase in C-cell adenomas in female rats. A review of the slides confirmed the presence of the adenomas but also revealed that contrary to the protocol specifications, the laboratory changed sectioning procedures for the thyroid during the tissue processing for the study. Since the controls had already been processed when the change was instituted, only the low dose and high dose animals had longitudinal thyroid sections which contained a greater proportion of the thyroid and hence more of these microscopic tumors (23). Because of this sampling bias, the marginal increase of benign tumors was not considered exposure-related (23).

The above examples all impacted on the incidence of tumors and in two cases, cancer rates in the controls. Verification that all lesions are included and tissue properly sectioned is essential for accurate historical tumor rates.

Review of Diagnoses

The pathologists involved in creation of the pathology data may function at one of three levels. The study pathologist is often involved in study design, in-life portion of the study, and the necropsies. The study pathologist evaluates all tissues and prepares a pathology report containing individual and summary diagnoses and a narrative placing the results in perspective. A Quality Assurance (QA) pathologist reviews the study and pathology reports and selected tissues to verify the results and identify potential problems. A panel of pathologists or pathology working group (PWG) that may include both the study pathologist and QA pathologist reviews slides, in a coded fashion, to resolve the issues raised by the study and the QA review. The use of QA pathologists and a PWG to assure diagnostic quality has been used for more than 20 years (3, 4, 8, 20), including for alternate species (2). In many cases, studies submitted to regulatory agencies now undergo some form of peer review prior to submission. While pathology reviews usually improve the accuracy and consistency of the diagnoses, unless care is taken such review may not identify and resolve problems and, in some cases, may actually introduce bias. This may occur when pathologists unfamiliar with concepts and practices of the QA and PWG process are asked to conduct a study review.

In large complex studies, errors will occur. Therefore it is crucial to review critical steps to identify and correct errors. One might assume that laboratories that have conducted studies for years would become very proficient and errors would be rare. However with changing personnel and evolving technology, some problems are found in nearly all studies. The NTP uses a 2-step process, a QA review to identify potential issues and PWG evaluation to resolve any issues raised by the QA review. For the first step, the NTP evaluates the study reports; individual animal diagnoses tables, clinical pathology results, and summary pathology tables. The literature on the chemical or chemical family is reviewed for expected toxicities. The tissues where toxicities are expected are selected for the QA pathologist's review. The pathology data for the control animals is carefully evaluated for incidence rates that differ markedly from historical controls and for unusual terminology or inconsistent terminology across study groups. This pathology data review can suggest additional tissues for review in addition to tissues that exhibit positive or negative trends in cancer rates. Occasionally excessive subcategories can mask a potential treatment related effect. Based on the pathology report and the pathology data review, the slides and lesions are selected for the QA review.

It is impossible for a study pathologist, not knowing which tissues may contain subtle effects, to examine 16,000 tissues (single species, 3 dose groups and control) with complete accuracy and consistency. The pathology evaluation may take 6 months or more and some diagnostic drift may occur. Recording errors, such as an obvious neoplasm not appearing in the final tables, occurs with every study. The QA pathologist, with a focus on treatment-related lesions and with a restricted number of tissues for evaluation can conduct a review in a brief period. This eliminates diagnostic drift, improves consistency and identifies lesions that may benefit from a PWG review.

Historically, the QA pathologist evaluated all tissues from 10% of the animals selected at random plus all tissues from all animals in which both positive and negative treatment-related trends occurred. We have found, however, that this is not necessary and not as effective as a more focused review on selected tissues related to specific issues identified by the study and data review. The QA pathologist provides a report detailing the agreements and discrepancies with the study pathologist. It is crucial to note that the QA pathologist is not providing a reread of the study and does not review slides in a coded fashion. The QA pathologist focuses on the existing study diagnosis for consistency and accuracy with a selected review of tissues for undiagnosed lesions.

The next step is the PWG review. The PWG provides a qualitative assessment of the lesions, assures diagnostic consistency, and provides confirmation of study results by a panel of experts. The QA pathologist may also serve as the PWG chair but for most NTP reviews, a third pathologist serves as the PWG chair. The PWG chair evaluates both the study and QA pathology reports. He/she then evaluates slides with discrepancies and selected treatment-related lesions for which both study and QA pathologist agree. The chair determines whether the appropriate tissues were selected for the QA review or if more tissues need to be reviewed. The PWG chair identifies the issues to be resolved and envisions how the PWG will be conducted. The chair selects pathologists with the appropriate expertise, and from differing backgrounds, as PWG participants. A slide set that is representative and addresses issues raised by the study is selected for PWG review.

A common mistake for many PWG chairs is to select so many slides that adequate attention is not given to each case. The PWG process can not be used for study re-evaluation.

Only in unusual cases should more than 100 slides be selected for review. With more slides than this, multiple days may be required for the PWG and this may not be the most effective use of time and resources nor give the most reliable results. With several hundred similar discrepancies between QA and study pathologist, it is much more efficient and accurate to review a subset and clearly establish the criteria and terminology. The QA or study pathologist can then review, and update the changes in question, keeping the PWG comments in mind, without the time constraints of the PWG. The most effective use of the PWG is to gain informed opinions on selected lesions that are problematic and crucial to the study and to verify study results. When PWG members are asked to review a large number of slides with diverse lesions, the quality of the review is lessened.

The questions for the PWG participants must be focused. When two different diagnostic issues, for example, classification of neoplasia and the presence or absence of atrophy involve one tissue, it is more effective to have one slide set address neoplasia and a second slide set address the atrophy. The PWG chair serves an important role in limiting the scope and setting the agenda for the review.

On the day of the PWG, the chair has 6 to 10 experts most of whom are unfamiliar with the study and unaware of the task at hand. The chair has the opportunity to make the task exciting, informative, and useful. This does not always happen. The most effective chairs provide study information either prior to the PWG or on the day of the PWG. It is useful to know why the compound is under study, why the study is important, what issues have been raised and what is expected of the PWG review.

A recent PWG addressed whether it was feasible and reliable to subclassify lymphomas in a large lifetime mouse study. The study involved over 2,500 C57BL/6 mice, approximately 1,500 of which had leukemia or lymphoma. The animals had been held until moribund or dead and only H&E stained slides were available for most cases. The study pathologist had subclassified all of the mouse lymphomas/leukemias using the Pattengale classification (26). The QA pathologist provided each member prior to the PWG with literature, diagnostic descriptions, and color prints of cases from each subcategory. The question for the PWG was should the study be analyzed only for total lymphomas or were the morphological criteria for subcategories distinct and useful. The PWG first reviewed classic examples from each category with diagnoses provided. The PWG next reviewed representative examples of subcategory in a coded fashion. Once the PWG was familiar with the criteria and the lesions as it occurred in this mouse strain, the remainder of the day was spent reviewing cases in a coded fashion.

The PWG determined that there was excellent agreement between study, QA and PWG pathologists for lymphoblastic lymphoma, lymphocytic lymphoma, histiocytic sarcoma, and granulocytic leukemia. The subcategories of B-cell lymphomas (follicular center cell, plasma cell, and immunoblastic lymphoma) were difficult for the PWG to separate consistently. The PWG provided confidence in the study results. The PWG also determined that T-cell and B-cell lymphomas could be evaluated separately for an exposure effect but that the subcategories of B-cell lymphomas should be grouped for analysis (6). The organized approach taken by the PWG chair assured the success of the review process in a large and complex study.

In some studies, very few discrepancies are found and the task is to confirm an effect or lack of effect. The questions for the PWG members must be targeted and representative of the study results. The PWG members need to evaluate enough cases to ensure confidence in results remembering that only a small percentage of slides come are reviewed by the PWG. It is crucial that the slide set selected for PWG review is representative of the study results.

The PWG results must reflect the diversity of opinions. Forcing consensus detracts from the PWG process and is misleading. In some PWG reviews, there will be nearly unanimous consensus on the diagnoses of lesions. In that case, one can be confident that other pathologists would come to similar conclusions and there is a high confidence in the diagnoses. Equally plausible and informative is when there is considerable disagreement about the nature of the lesion in a study, for example, whether the treatment-related lesions are benign or malignant. There should never be an attempt to force a consensus. A single dissenting PWG member may call attention to an aspect of a particular lesion that upon further review will influence other members. All members should be encouraged to present their views, no one should be allowed to dominate, and the diagnoses and report should reflect the diversity of the opinion. When the PWG opinions are nearly evenly split, the NTP accepts the study pathologist diagnoses but acknowledges in the study report that the nature of the lesions is less certain.

The PWG review is only as good as the PWG chairperson and the participants. The chair must select a balance of pathologists with expertise in the area of question. We favor including both the study pathologist and QA pathologist when possible. The chair sets the tone of the review and presents the participants with the issues to be resolved by the PWG. It is often helpful for the initial set of slides to include normal, benign, and malignant lesions where both QA and study pathologist agree. This can be followed by a discussion of the criteria, terminology and what each member feels is critical for the diagnostic categories. Selected lesions should then be reviewed in a coded fashion. PWG members record their diagnoses, and then discuss each case. The PWG is an opportunity to be an educational process for the QA pathologist, the study pathologist, and the PWG members. It is a unique opportunity to evaluate a series of lesions in a short period and have the opportunity to compare diagnostic criteria and diagnoses with colleagues. The study director, stakeholders, and other interested parties can gain significant insight on the chemical effects by observing the PWG process. All participants gain in a process that improves and adds credibility to the study.

Review of the pathology materials and diagnoses markedly improves study quality at only a fraction of the cost of the original study. The review can be accomplished in several months and is necessary because chronic study results are essentially permanent. Although second and third studies can be done, it is essentially impossible to replicate a 2-year study since animals, rodent diets, and other conditions evolve during the time of a 2-year study. In contrast to short-term assays where multiple replications can prove or disprove a result, the

long-term rodent study results remain in the literature. Thus, getting the original results correct remains a priority.

The review works best when considered as a collaborative effort and not as a judgment on study conduct or pathology competency. Collecting and recording data consistently over 2 years including weekends and holidays is a difficult task. The initial pathology examination of over 16,000 tissues is arduous and subject to errors. Data auditors, QA pathologists, and PWG members are all part of a collaborative effort to assure the best possible data. The QA and PWG pathologists when serving as study pathologists are subject to the same inherent limitations as the study pathologist whose data is being reviewed. Because many pathologists may function in different roles in different studies, most are sympathetic with the difficulty of the study pathologist's task and recognize the need for peer review for all studies.

The NTP Database

The NTP historical control database consists of all studies carried out within a time window of 5 to 7 years for which the diagnoses have been finalized following a QA and PWG review. Approximately once a year, newer studies are added to the database and older studies are deleted to ensure that the neoplasm rates are consistent with current experience. Historical control summary reports are prepared that provide study-by-study tabulations of tumor rates for all site-specific tumors and certain tumor combinations. Periodically, these tabulations of tumor rates are published in the peer-reviewed literature (11–15) in order to make such data more readily available to the scientific community.

A number of factors may influence tumor occurrence, and to the extent possible, the historical control report takes these factors into account (16). For example, separate tabulations are prepared for each sex, strain, species, study duration, and type of control group (eg, untreated controls from feeding studies, chamber controls from inhalation studies, corn oil gavage controls from gavage studies). Separate tabulations are given for the new NTP 2000 diet and the older NIH07 diet. The laboratory conducting each study is identified, so that lab-to-lab variability in tumor incidence can be assessed. Finally, summary values for survival and body weight are also provided in this report, because both of these factors are known to influence tumor occurrence.

Role of the Pathologist in Toxicogenomics

Pathologists are now being included in teams evaluating gene expression patterns found after exposure to xenobiotics. This application of the genomic technology to toxicology research has been termed toxicogenomics. Current differential gene expression (DGE) platforms can detect differences in as few as one or two transcripts of a particular gene. Because of this remarkable sensitivity, small shifts in cellular populations exert marked influences on gene expression patterns. Pathologists are often asked to evaluate cells or tissues being analyzed by DGE. The pathologist can help interpret the interplay between the molecular biology and biochemistry of the specimen as presented in the DGE and the morphologic alterations. However, this requires an investment by the pathologist in understanding basic intermediary metabolism and biochemistry, as well as the molecular biology of transcriptional controls.

The pathologist may provide critical input for the toxicogenomics team in several areas beyond the standard morphological support. The pathologist being familiar with animals and tissues that serve as RNA and protein sources for toxicogenomic studies can inform and help control for factors that may impact study results. Animal health, sex, housing, diet, circadian rhythm can all affect DGE. The pathologist can assist in selection of tissue for study because other team members may not be aware of the substructure of a tissue. For example, the RNA or protein needs to be isolated from the same level of renal cortex or from the same liver lobe for each group.

Toxicogenomics research teams are interested in correlating DGE with pathology phenotypes. Is there a signature gene profile that can be correlated with preneoplastic lesions? It is crucial that the pathologist use the same care in assuring the diagnoses as would be done in a standard rodent study. We suggest that pathologists use similar QA and PWG processes to assure that different toxicogenomic studies are comparing similar pathology phenotypes. The genomic revolution will have a dramatic effect on the practice of pathology (1). It is essential that pathologists become more involved in the molecular interpretation of differential gene displays and in the design of studies that fully utilize the power of molecular biology in the context of classical pathology.

Pathology Data for Toxicogenomic Databases

The NIEHS National Center for Toxicogenomics (NCT) is developing a database to link DGE with pathology tables and histopathology image files. This database will be called the Chemical Effects in Biological Systems (CEBS) database. A central tenant of this toxicogenomics database is that it will be informative to compare changes in gene and protein expression resulting from chemical toxicity to clinical history, hematology, clinical chemistry, and histopathologic images and descriptions. All such information will be linked in a toxicology experiment as a function of test chemical, dose, time, and specific gene or protein identification. The process of gene (and protein) annotation resulting from toxicogenomic experimentation will be resolved further into the associated biological pathways and networks such that mode(s)-of-action can be derived for the toxicologic and pathologic effects encountered with a given exposure. Panels or compendia (18) of genes and proteins associated with particular pathways and outcomes (eg, pathologies) will be defined and documented with appropriate data or digital images. CEBS will become a public resource and knowledge base for the documentation of chemical effects on biological systems. It may be possible to distinguish homeostatic or compensatory responses to chemicals from adverse or toxic responses. Such information should prove invaluable in protecting the public health and for risk assessments on environmental exposures. It seems obvious that the same pathology review procedures used to ensure the integrity of historical databases should be applied to the pathology images that become part of this database.

Conclusions

In the past 20 years, the NTP has refined and streamlined a process to provide comprehensive review of the pathology data and diagnoses from NTP studies. The pathology review

is increasingly recognized as a cost-effective procedure to improve accuracy and facilitate comparison between studies. A thorough review of the study results and careful decisions on the QA and PWG approach is important. Simply applying a set review procedure without careful judgment on how to avoid bias in the process or forcing PWG consensus when one does not exist will not ensure study quality.

A second evaluation of a study in a coded fashion is not as an effective process in most cases. The second pathologist has to evaluate each slide as was done by the study pathologist. This takes essentially the same time and provides a second and different opinion that may not be any more valid than that of the study pathologist. A third review results in 3 differing data sets that may be of similar quality with no progression to resolution. The QA and PWG process that we have described is a focused hierarchical process that leads to resolution of issues and increased confidence in the study results.

Pathologists are now often involved in an exciting genomic revolution with RNA and protein expression profiling being used for drug discovery and chemical safety assessment. Pathologists as members of diverse research teams have the opportunity to communicate the significance of the pathology results and digital images make sharing data easier. Digital pathology images are being included in relational database linked to gene expression changes. Pathologists can use the review QA and PWG review process to assure accuracy and uniformity of data for the toxicogenomic databases. Lessons learned in assuring pathology data quality in standard rodent studies should not be forgotten.

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